OBSERVATIONS ON DOUBLY LIGATED CAROTID ARTERIES IN THE RABBIT

GEORGE A. CARDEN, JR.

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George A. Carden, Jr., Ph. B.,
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Department of Pathology
School of Medicine
Yale University

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OUTLINE

I INTRODUCTION

Many approaches have been made to the study of arterial disease, but few have yielded any contributions toward a better understanding of the fundamental underlying factors involved.

A few years ago, Dr. Raymond Hussey became interested in the subject and soon came to the conclusion that if any advancement were to be made it must come as a result of a more thorough understanding of the anatomy and physiology of the vessel wall and, in particular, of the nutrition of the intima and inner two-thirds of the media.

In the course of another experiment, Dr. Elizabeth M. Ramsey[86] observed that the lumen of arteries became occluded by a mass of connective tissue following long-time double ligation. As our interest in determining the nature of this mass grew, it became evident that a procedure might be designed which would not only throw some light on the origin of the mass in the lumen of long-time ligated vessels but also possibly add something to the all important question concerning the source of nutrition of the intima and inner two-thirds of the media

The present experiment was therefore designed:

1) to eliminate the possibility of thrombus formation within the ligated stretch,
2) to isolate the vessel from all possible sources of blood supply, and
3) to study the effects of this procedure on all three coats of the vessel wall at varying intervals of time from a few days to several weeks.

II LITERATURE

No attempt will be made to review the vast literature on arterial disease, as it has no particular bearing on the present paper. Furthermore, it has been adequately dealt with in a recent publication by Cowdry[1] under the auspices of the Josiah Macy, Jr. Foundation. Likewise, no attempt will be made to review the literature on the anatomy and physiology of blood vessels, as that will be dealt with in the full report of the investigation of which this paper is only a part. It might be well, however, to review briefly some of the experimental work on this subject, in order to orient the reader with respect to what has been done before, as well as to correlate the problem as a whole with our findings.

The question of the source of nutrition of the walls of arteries and veins has been a controversal one from the time this subject first became a concern to anatomists, physiologists and pathologists up to the present day. It was easily and adequately demonstrated very early by many that there are blood vessels, both arterial and venous in the adventitia of both arteries and veins, which presumably supply this portion of the vessel walls. It was also conclusively shown by Risse[2] and others that these

vasa vasorum normally penetrate to the outer layers of the media. At this point the controversy arose - was the intima and inner two-thirds of the media supplied by the vasa vasorum, and if so, directly or through the medium of lymph channels into the vasa vasorum? Or were they supplied through the lumen, and if so was this by means of blood channels leading from the lumen? Or by means of a diaphoresis through the endothelial cells and thence through the fenestrations of Henle in the internal elastic membrane? Or, finally, was the nutriant supply obtained through a combination of these mechanisms? Quite naturally there were numerous adherents to each school, and each with clinical, pathological or experimental observations, all of questionable accuracy and frequently full of imaginative interpretations, to support his particular view. However, the consensus of opinion soon swung to Risse's[2] original contention on the basis of injection experiments, that anatomically demonstrable blood vessels can only be seen in the adventitia and outer layers of the media, though he feels that the remainder of the vessel wall must be supplied through the vasa vasorum by minute capillaries or through a lymph canal system. For how else, he asks, can one explain the integrity of the vessel wall following on periods of occlusion of the lumen by thrombus? This view likewise receives the support of Köster,[3] Renaut,[4] Martin,[5] Huebner,[6] de Giovanni[7] and his pupil, Frigo,[8] and others, who on the basis of anatomical and pathological studies believe that

diseases of the intima are secondary to diseases of the vasa vasorum.

Frigo's[8] work on this subject is particularly illuminating. He made transverse and longitudinal sections of arteries of individuals, who in life showed no symptoms of arteritis, and at autopsy showed only small patches of atheromata in the large arteries. In the sections containing the atheromatous scars, he found the intima of the vasa vasorum thickened and uneven and projecting into the vessel in such a way as to lessen its lumen by a third or a half. He found this intimal proliferation at times composed of a homogeneous basic substance with many cells, some round, o others more prolonged or fusiform; and at other times the cells were few and replaced almost entirely by a fibrillary substance. From these observations it seemed clear to him:

1) that the change represented a proliferation of the endothelium of the vasa vasorum, which had caused thickening of the intima and diminution of the vascular lumen, and
2) that this was a clear pathological proof of the theory that lesions of the vasa vasorum are the primary factors in the pathogenisis of arteritis and endarteritis.

He furthermore showed degenerative and proliferative lesions of the kidney, myocardium and sympathetic nerve ganglea, in which there was an easily demonstrable, partially occluding endarteritis of their nutrient arteries. This convinced him that the great bulk of arterial lesions seen in elderly people was, in its fundamental process, entirely analagous to

the degenerative lesions of the organs supplied by these vessels, and that the whole process could be explained on the basis of a diminution of the blood supply to the tissue in question.

Following these extremely interesting observations of Frigo[8] and de Giovanni[7], which received the support of other members of their school mentioned above, one would have expected to find the literature fairly abundant with interesting animal experimentation attempting to further elucidate tha part played by the vasa vasorum in arterial lesions. Such, however, is not the case. With two exceptions, which will be taken up in detail later, the approach to the problem has been persued along four lines: I Infections and Toxins, II Changes in Blood Pressure, III High Cholesterol Diets, and IV Chemical and Mechanical Injury.

I Infections and Toxins.

The literature is abundant with descriptions of various vascular lesions, both pathological and experimental, seen following infections and toxemias. The effect of syphilitic infection on the arterial system, first emphasized by Huebner[6] and confirmed and elaborated upon by Welch[9], Döhle[10], Haller[11], Benda[12], Schmorl[13] and many others, is still an established fact, but does not serve in any way to explain the great mass of arterial lesions in which there is no relationship to syphilis. The literature is abundant with descriptions by Thérèse[14], Huchard[15], Jores[16], Wiesel[17], Klotz[18], Frothingham[19]

Faber,[20] Aschoff,[21] and more recently by Ophüls[22] of autopsies of individuals dying of various infectious diseases, in which they find arterial lesions ranging from inflammation and proliferation of the intima to necrosis and calcification of the media. However, MacCallum,[23] on the basis of an elaborate statistical study as well as a careful review of the subject, concludes that there is but little evidence in favor of the idea that infections, acute or chronic, play a great part in the pathogenesis of arteriosclerosis.

Likewise there are many descriptions of experimental production of arterial lesions by living or dead bacteria or filtrates of cultures. Gilbert and Lion[24] produced scattered sclerotic and calcarious changes with the injection of bacteria and toxins, as also did Thérèse,[14] Crocq,[25] Boinet and Romary,[26] and Manouélian.[27] Klotz,[18] working with a large series of animals, found that typhoid and streptococcal infections produced primarily proliferative changes in the intima with little change in the media; while with the injection of diptheria toxin he and Bailey[28] produced distinct medial lesions with calcification. Salteykow[29] observed thickenings of the intima with deposits of large masses of fatty substances in the proliferated tissue following the intravenous injection of staphlococci and the introduction of alcohol. Attempts by Fahr,[30] Starokadomsky and Ssobolew,[31] Redingius[32] and others to confirm these observations were unsuccessful.

The lack of uniformity in all of this work on the possible effect of infections and toxins on the etiology of

arterial lesions is strong contradictory evidence to the theory that these factors play any primary role in the pathogenesis of arterial disease.

II Changes in Blood Pressure

The advent of a paper by Josué[33] in 1903, in which he reported that he had artificially produced "atheroma" in the aortas of rabbits by intravenous injections of adrenalin, was hailed by many as the beginning of a new era in the study of vascular disease. It was soon followed by reports by Erb,[34] Külbs,[35] Ziegler,[36] von Rzentkowski,[37] Fischer,[38] Scheidmantel,[39] D'Amato,[40] Pearce and Stanton,[41] Lissauer,[42] Baylac and Albarede,[43] Biland[44] and others, who in the main confirmed Josué's[33] work. These lesions, which are primarily necrosis and degeneration of the media with excessive calcification, without conspicuous changes in the intima, and which resemble the so-called Monckeberg type of sclerosis seen in human arteries, were so constant in their pathology that they soon received the name of 'adrenalin sclerosis'.

The enthusiasm for this discovery began to wane, however, when it was found that a number of other substances, some of thich raised the blood pressure, others which lowered it and still others which had no effect on it, all produced the same lesion. For example, positive results were obtaimed with digitalin (Fischer),[45] with barium chloride (Miller),[46] with barium chloride, hydrastin and hydrastinin (Bennecke),[47]

with methylamino-acetobrezkatechin (Sturli)[48], with nicotine (Adler and Hansel[49], Baylac[50], and Rickett)[51], with euthalmin (Mironescu)[52], with amylnitrite and adrenalin together (Braun)[53], with phloridzin and phloretin (Kolisch)[54], with potassium iodide (Hedinger and Loeb)[55], even with acids, ferments and normal saline (Fischer)[45], and this is only to mention a few. Furthermore it soon appeared that repeated attempts to produce similar lesions in other animals, such as dogs and monkeys, invariably failed.

The final bombshell came with a paper by Miles who reported spontaneous lesions precisely similar to the so-called 'adrenalin sclerosis' in nearly 35% of presumably normal animals. This observation was amply confirmed by Hill,[57] Meyers,[58] in part by Pearce[59] and by Ophüls.[60]

III High Cholesterol Diets

The disappointing results obtained by the investigators attempting to account for the pathogenesis of arterial disease on the basis of: I, Toxins and Infections and II, Changes in Blood Pressure led Ignatowsky,[61] in 1908, to attempt a new approach to the problem. He fed large quantities of meat, eggs and milk to rabbits and observed intimal thickenings in the aorta somewhat similar to arteriosclerosis in humans. He therefore concluded that it was the excess protein in their diet which produced these changes. This experiment was successfully repeated by Fahr[62] in 1912, but Stuckey[63] in the same year and Wesselkin[64] a year later showed conclusively

that these changes were not due to the protein, but rather to certain fatty substances in the diet. At the same time Anitschkow,[65] independently, and working with Chalatow,[66] produced this so-called rabbit arteriosclerosis by feeding the animals pure cholesterin and vegetable oil. This new approach was immediately taken up by Wacker and Hueck,[67] Kon,[68] Aschoff,[21] Waristscheff,[69] Bailey,[70] McMeans and Klotz,[71] later by Schrönheimer[72] and many other investigators. They amply confirmed and further elaborated the original observations of Anitschow[65] and Chalatow[66] that cholesterin, in combination with oil, given to rabbits by mouth produces in the aorta a lesion on the inner coat of the vessel very similar to the nonulcerated atheromatous plaques so frequently observed in human material.

These changes are uniform, most prominent in the aortic arch, in the semilunar valves and in the abdominal aorta. The early changes, within the first 20-30 days as described by Zinserling[73] and others, consists of the deposition of finely granulated lipoid material:

1) in the interstices between the individual elastic lamellae, or between the individual muscle fibers of the innermost layers of the media, and
2) between the endothelium and the internal elastic membrane, raising the single layer of endothelial cells from the internal elastic membrane.

Later on in the process two other changes take place:

1) mononuclear cells containing globules of lipoid material appear between the endothelium and the internal elastic lamella and increase until a thick layer of them is formed on the internal elastic lamella, and
2) a splitting off of the individual fibrils of the internal elastic lamella toward the intima until this membrane becomes thin and fragmented in places and the proliferated intima contains a dense network of elastic fibrils.

Finally the gaps in the internal elastic lamella become invaded with groups of muscle cells from the innermost layers of the media. Some of these cells contain lipoid material.

The process is apparently a diffuse one, for these changes have recently been found in the coronary arteries (Wolkoff)[74], in the pulmonary and carotid arteries (Bailey)[70] and even in the larger veins (Schöenheimer)[72]. Furthermore, it has been found in many of the smaller arteries (Versé)[75], and also in the sclera, cornea and ciliary body of the eye (Versé[76] and Kolen)[77]. This latter finding is of particular interest in view of the question which has frequently been raised as to whether the intima and inner two-thirds of the media is not analagous, in the physiology of its nutrition, to the cornea and sclera of the eye.

In connection with observations on the absorption of the lipoid material in such lesions, Anitschkow[78] turned to the use of colloid stains in an attempt to elucidate the pathways of absorption of the lipoid material, and further

to throw some possible light on the persistently opaque subject of nutrition of vascular walls in general. He concluded from this work:

1) that the material reaches the intima through the lumen,
2) that in the process of absorption it is carried through the media and emptied into the lymphatics in the adventitia,
3) that under normal conditions there is a constant flow of lymph from the lumen to the adventitia.

Similar conclusions were reached by Petroff[79] and Okuneff[80] by the use of trypan blue stain. However, with respect to the mass of material pointing to the impregnation of lipoid material in the intima as the primary cause of atheromata, Anitschkow has wisely said that there must be some other factor, in the production of the lesion, which still eludes us. Aschoff believes this elusive factor to be wear and tear or escessive stresses causing a loosening of the ground substance of the vessel.

IV Chemical and Mechanical Injuries.

The application of strong solutions of silver nitrate to the vessel wall of the aorta, as shown by Hill[57] and others, produces after a protracted period of time a fibrous thickening of the adventitia and a calcification of the outer portion of the media without significant changes in the intima or the inner two-thirds of the media.

Mechanical trauma, as by crushing, pinching, pulling and so forth, is seen to give rise to a proliferation of the intima, fraying of the internal elastic lamella, a proliferation of the cellular parts of the media with an invasion of these cells into the intima through the breaks in the internal elastic lamella. If the injury is sufficient it may produce atrophy of the media and adventitia with replacement by granulation tissue. Ssolowjew's[31] experiment is particularly illuminating on this subject. He has shown that isolated tears in the internal elastic membrane results inaa hypertrophy of the intima at that point, and furthermore that this area shows an increased permeability to vital dies. He demonstrated another interesting phenomena, namely that displacement of the carotid artery of a rabbit on to a bridge of skin will result, not immediately but eventually, in breaks in the internal elastic lamella with cells of the media growing through these breaks into the proliferating intima. To control this observation he produced trauma to the opposite vessel of a distinctly more severe character than the operation of the bridged vessel entailed and found no changes in the vessel so traumatized. He was, therefore, convinced that some factor other than trauma was of prime importance in the production of these lesions, and he suggests as a possibility the fixation of the surrounding artery by fibrous tissue.

The question of the effect on the vessel walls of the injury to their innervating nerves was first introduced by de Giovanni[7] and Frigo[8] as a result of their study of human pathological material and of a limited amount of research on dogs. The weight of evidence, however, has not been support of these early views of de Giovanni[7] and Frigo[8] on the effect of nerve injury on the vessel. "Lewaschew, Frankel and Barvoets produced extensive lesions in vessels by resection of nerves (sciatic nerve). Von Czyhlarz and Helbing have conclusively shown that the lesions produced by these observers are of a purely inflammatory character and will not ensue if all inflammation is prevented."[82] Buerger[83] says, in relation to the etiology of thrombo-angiotos obliterans, that the nerve lesions are secondary and apparently dependent upon the fibrotic perivascular changes.

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We now come to a consideration of the two exceptions to the four methods of approach to the experimental study of vascular disease outlined above. In this connection, a paper by Baumgarten[84] in 1876, and one by Schaeffer and Radasch[85] in 1924 will be taken up in some detail, in view of the pertinent bearing the work of these two men has on our present investigation.

Baumgarten,[84] to study the pathogenesis of thrombus formation, performed the following experiment: he doubly ligated the arteries and veins in rabbits by dissecting free*

* He did not state whether or not he ligated intervening branches encountered in the dissected stretch.

an inch long stretch and suddenly stopping the circulation by application ot two ligatures. At varying intervals following this procedure, he observed in the sections between the ligatures a cellular proliferation on the intima of the arteries, without (with the exception of the regions close to the ligatures) any evidence of infiltration or proliferation in the media and adventitia.

The first change noted by him was a filling in of the troughs of the internal elastic lamella with nuclei, large and small, which raised the endothelium above the internal elastic lamella. As time passed, the cell mass increased in extent, and in its growth narrowed the lumen. Along with this he observed a differentiation of the newly formed tissue elements. The cells near the lumen he described as elongated and spindle-shaped and arranged in tiers, which he believed to represent a newly formed muscle layer (but without any picric acid reaction which further on in his paper he eluded to as fibroblastic proliferation). The outer stratum of the proliferating intima contained what he referred to as radial streaming cells forming a loose irregular network.

As this process progressed, he found the lumen narrowed at times to pinpoint size, but no further metamorphosis of the newly formed tissue occured - that is to say no vessels appeared in it.

While the above changes appeared in the ligated stretch between the ligatures, he observed another process

at the points of ligature. Here the threads of ligature appeared to have ruptured all three coats of the vessel wall and in this region a richly vascular connective tissue proliferation was observed which extended in both directions for a varying distance beyond the site of the ligature.

He summarized his results as follows:

"The so-called organization of red thrombi....

...takes place by means of two independent processes:

a) By proliferation of the vascular lumen.

b) By proliferation of tissue at the points of ligation which penetrates inward and to which alone I ascribe the formation of new vessels.

" The clot plays a zero role in the process of organization......This estimate of its role is the result of the observation that the process of organization can take place when the blood is entirely removed from the lumen". *

He does not describe the experiments in which the blood was removed from the lumen.

The remainder of his paper is taken up in a discussion of the origin of the cells in the intimal proliferation, which we will refer to in our DISCUSSION.

*Translation.

Schaeffer and Radasch,[85] using a series of forty-five rabbits, doubly ligated one carotid artery in each animal, and studied sections of the vessels after various ligation intervals, ranging from 6-92 days. They placed the distal ligature first in order to insure a blood-filled segment between the ligatures.

In one series of their vessels, they found thrombosis a common occurence, while in another series its occurence was rare. In the cases where organization of a thrombus had taken place, there was a relative abundance of elastic tissue in the vascularized granulation tissue.

When thrombosis did not take place, a gradual orderly progressive thickening of the intima was observed, until the lumen was completely obliterated.

The early changes observed by these men consist of:

1) A swelling and desquamation of the endothelium.
2) The appearance of a fibro-cellular material filling the troughs of the internal elastic lamella, and
3) A crowding together and piling up of the endothelial cells.

At later stages they observed the appearance of delicate, straight fibrils of elastic tissue proliferally placed next to the internal elastic lamella.

When the lumen had become narrowed to one half or more of its original size by the proliferating intima, they observed a zoning of the cells, consisting of a thick

stratum of fibroblasts just beneath the intact proliferated endothelium, and beneath this a broad band of loosely arranged spindle and round cells merging into a somewhat denser proliferal zone. By the use of an elastic tissue stain, they demonstrated a great amount of elastic tissue in the growing intima. In the photomicrographs which accompany their paper, the elastic tissue appears to arise in loops from the internal elastic lamella and in some places has a tendency to form thin lamellae. However, they state in their text that the fibrils are fine, short and almost straight.

They conclude from their observations that, in the obliteration of the lumen between ligatures in the common carotid artery of rabbits, three processes may be taking place:

"1) Thrombosis with secondary organization,

2) A ring-like thickening of the intima by the formation and continued growth of a sub-endothelial stratum, and

3) A combination of these.

Of these processes, the thickening of the intima appears to be the most important factor."

They also note that the thickening of the intima is frequently more marked on one side, giving rise to an eccentric position of the remaining lumen.

III METHODS AND MATERIAL

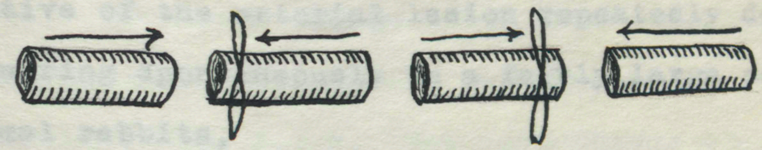
The carotid arteries of rabbits were the experimental subjects employed. Only adult rabbits were used, averaging 5-7 pounds. No one species of rabbit was used exclusively, and members of either sex were used indiscriminately. The animals were fed on average, well-balanced ration and were kept in cages in an airy room.

The animals were anesthetized with sodium-ethyo (methyl-butyl) barbiturate (pentobarbital sodium) and their necks shaved and prepared with alcohol. Sterile drapes were applied and the skin, subcutaneous tissues, and muscles were incised in the midline down to the trachea and retracted laterally, exposing the carotid sheath with its contained structures. The sheath was opened and the artery completely freed from all surrounding fascia and connective tissue from the level of the upper portion of the larynx to the sternal notch. Any branches of the artery encountered in this stretch were doubly ligated and cut.

The proximal ligature was applied first at the lower end of the isolated stretch of the vessel, the blood was then gently milked from the vessel and the distal ligature tied at the upper end of the

isolated stretch. Great care was taken in every case to traumatize the vessel as little as possible. Before closing the neck by interupted silk sutures in the muscle, fascia and skin, careful observation was made without probing to see if the vessel between the ligatures continued to remain bloodless.

When the specimens were removed at the desired intervals after the initial procedure, they were fixed in Zenker-Acetic solution and carried through the usual fixatives. Only one vessel of each animal was used for experimental purposes. The contralateral vessel, in each case, was removed as a control. After fixing, the specimens were cut into blocks embedded in paraffin and sectioned so as to obtain sections above, between and below the ligatures, according to the following diagram:



The sections were routinely stained with hemotoxylin and eosin, Weigert's elastic tissue stain and Mallory's connective tissue stain.

IV EXPERIMENTAL RESULTS

The sections of the controls in each case are found to be entirely uniform. The internal elastic membrane is completely intact, Fig. 1,2. The intima is composed of a single layer of endothelial cells, linked by thin strands of protoplasm and closely applied to the internal elastic membrane. There is no evidence of there being any tissue elements between the endothelium and the internal elastic membrane, for the former can be seen to be dipping down into the troughs and riding the crests of this undulating membrane, as is well shown in Fig. 2. The media and adventitia are likewise uniform throughout the control series and in no case were any changes seen suggestive of the arterial lesion repeatedly described as occurring spontaneously in a fairly large percentage of normal rabbits.

In the experimental series, the procedure was devised to study the effects produced when a vessel is isolated from its blood supply, both by way of the vasa vasorum and the lumen, for a protracted period of time. Seventeen arteries were subjected to simple double ligation for the following periods of time: 4, 7, 16, 21, 26, 29, and 38 days.

The result of this experimental procedure is a proliferation of the intima, without any detectable disturbance in the media or adventitia. Wide variation was found in the extent of the proliferation occurring following different periods of ligation. However, this variation did not strictly parallel the duration of ligation. Nor did two arteries, ligated for identical periods of time, necessarily show identical changes. For example, one artery, ligated for 26 days, shows changes no more advanced than those in an artery ligated for 16 days. While another 26 day vessel shows quite marked changes. (chart) Despite these discrepancies, it can safely be said that, in general, vessels ligated for a short period of time do not show changes as extensive as those noted in vessels which were ligated for a longer time.

The first change noted is a crowding together and a piling up of endothelial cells upon the internal elastic lamella, Fig. 3,4,5. The next change observed is a streaming out of cells which elevate the endothelium above the internal elastic lamella, Fig. 5,6,7,8,9. As these cells grow out, they tend to form loops, one upon the other, using the internal elastic lamella as their base, Fig. 5. With Mallory's connective tissue stain, the nuclei of these cells are red and the cytoplasm is blue. The possibibities concerning their origin will be taken up in the DISCUSSION.

This intimal proliferation does not take place uniformly, but is eccentric in the majority of instances and varies in extent at different levels between the ligatures. Thus we see in the early stages (1-2 weeks) a fibrilo-cellular network of collagenous material, arising between the proliferated endothelium and the internal elastic lamella, Fig. 5,6,7. When the process has reached a stage at which the lumen is reduced to two-thirds its original size, an elastic tissue stain demonstrates thin strands of elastic tissue growing out from the region of the internal elastic lamella into the proliferating intima, Fig. 10. At a later stage, this elastic tissue is found to be distributed in lamellae very similar to those found in the media, Fig. 11. As the narrowing of the lumen progresses, the endothelial cells become more crowded together, the connective tissue just beneath the endothelium becomes denser as well as that adjacent to the internal elastic lamella, while the intervening tissue appears as a loose network of collagenous material with relatively few nuclei. The characteristic appearance of a vessel at this stage (3-5 weeks) is therefore that of a central band of endothelial cells, 3-5 cells deep, bordering upon a small lumen and surrounded by an intermediate zone of a fibrillar, sparsely cellular network which merges into a dense zone of richly cellular

collagenous material, closely applied to the internal elastic lamella, Fig. 8,9,10,12, 13.

The two vessels which were ligated for 29 days show a change which is not duplicated in any of the other specimens. The lumen is completely filled with loosely arranged fibrous connective tissue, in which are noted numerous small capillaries and well formed arterioles containing red blood cells. In addition, sections prepared with Weigert's stain show fragments of elastic tissue throughout the tissue filling the lumen. In these specimens there is no semblance of the orderly proliferative process described above and noted in all the other sections which were taken between the ligatures. Fig.13,15.

In an attempt to clarify the problem raised by these two specimens and to relate them to the others described and further to explain the appearance of varying degrees of intimal proliferation at different levels of the same vessel, a longitudinal section was prepared of another 29 day specimen. In this specimen, the central portion between the ligatures shows a condition similar to that observed in cross sections of 21, 26 and 28 day vessels. Close to the ligature at one end of the ligated stretch, the lumen contains a plug of loosely arranged fibrillar connective tissue, which is richly vascular, as in the two 29 day specimens described, Fig. 16, 17, 18.

Though it is dangerous to generalize on one observation, nevertheless the evidence which this one longitudinal section presents as an explanation to a number of heretofore obscure points is rather convincing. It shows that the ligatures inflict severe injury to all three coats of the vessel wall, that this injury has given rise, in this case, to an organized thrombus at one of the points of ligature and furthermore that this organizing process fills the lumen of the vessel for a short distance on either side of the ligature. This material is very similar to that seen in the 29 day specimens described, and the inference is that the process is the same. At the same time, this longitudinal 29 day specimen demonstrates another process between the ligatures, a proliferative change, similar to that seen in all the other sections of this series (with the exception of the other two 29 day specimens), a proliferation which is orderly, though not concentric, free of capillaries: an avascular proliferation which is very different from that of an organized thrombus.

The accompanying chart serves (p.29) to summarize the findings in all the sections with the exception of those from the one 29 day vessel which was sectioned longitudinally and which has just been considered in some detail. Each observation as recorded on the chart represents the

sum total of observations on a number of sections. For example, a minimum of 30 sections were taken between the ligatures in every vessel - that is, 10 with each of the three stains (hemotoxylin and eosin, Weigert's and Mallory's) - and about 15 above and 15 below the ligatures - 5 with each stain. In some cases serial sections were taken, in addition. In practically every case the observation was recorded which showed the maximal amount of change, unless this appeared to be due to some artefact.

From the data in the first column, it is evident that the greatest proliferation occurs between the ligatures. The variability of the degree of proliferation, with relation to the ligation period, is clearly shown, by comparing the 16 and 26 day vessels and comparing the differance in degree of change in the four 21 day vessels.

The character of the proliferation is nodular and eccentric, for the most part.

The proliferation of endothelial cells is prominent in the early changes, but does not keep pace with the later changes.

The amount of connective tissuē seen, however, is progressive with the degree of intimal thickening.

Elastic tissue does appear in the proliferating intima as early as 21 days and is a constant finding between the ligatures in all of the later sections.

Capillaries are seen in the intima between the ligatures only in the two 29 day vessels mentioned. They are only seen once above and once below the ligatures. From the observations of the longitudinal sections, one would expect to see more evidence of organized thrombus above and below the ligatures. Their relative absence may simply indicate that the sections were not taken sufficiently near the ligatures to demonstrate the presence of an organizing thrombus at that point.

The observations concerning the amount of blood in the lumen are not reliable, for any or all of it may have been lost in the process of preparing the microscopic sections. It is interesting, however, that on two occasions the lumeh is fairly full of blood, and this in spite of every care to empty the vessels of all contained blood before tying the second ligature. The origin of this blood is not apparent from the sections. There is distinctly less blood in the sections between the ligatures than there is above and below.

With respect to the presence of blood pigments in phagocites between the ligatures, as would be expected, they are only seen in the case of a 29 day vessel, in which there are eapillaries and other evidences of an organized thrombus. They are also seen above and below the

ligatures in the two specimens in which capillaries are also seen in the intima.

In about half of the cases, careful search reveals breaks in the internal elastic lamella in one or more sections taken between the ligatures, and in some of these connective tissue cells appear to be growing from the innermost layers of the media into the proliferating intima through the breaks in the internal elastic lamella. This is well illustrated in Fig. 13.

It would therefore appear that two processes may take place as a result of simple double ligation:

1) A thrombus formation at the point of ligature, probably the result of two factors, injury to the vessel wall at that point and slowing of the stream of blood, and

2) A second process, the one most prominent in these slides, a fairly simple avascular intimal connective and elastic tissue proliferation, preceeded by an early response of the endothelial cells which does not appear to keep pace with the fibro-elastic proliferation.

DURATION of LIGATION	INTIMAL PROLIFERATION			CHARACTER of PROLIFERATION			PROLIFERATION of ENDOTHELIAL CELLS		
	Above	Between	Below	Above	Between	Below	Above	Between	Below
4 days	*	±	±	*	N	N	*	+	±
	0	*	±	0	*	N	0	*	±
7 days	0	±	±	0	N	N	0	+	±
	±	0	0	N	0	0	±	0	0
16 days	+	+	±	NC	C	C	+	+	+
	±	0	0	C	0	0	+	±	0
21 days	+	±	+	NC	NC	N	+	+	±
	+	+	±	NC	NC	N	+	+	±
	±	+++	0	N	NC	0	±	+	0
	+	+++	+++	NC	C	C	++	±	+
26 days	0	+++	+	0	NC	NL	0	+	+
	+	+	+	N	N	NC	+	+	+
29 days	±	////	±	N	NC	N	±	+	±
	±	////	////	N	NC	////	+	++	++
38 days	0	+++	*	0	NC	*	0	0	*
	++++	+++	*	////	NC	*	+	+	*

DURATION of LIGATION	PROLIFERATION of SUBENDOTELIAL CONNECTIVE TISSUE			ELASTIC TISSUE in INTIMA			CAPILLARIES in INTIMA		
	Above	Between	Below	Above	Between	Below	Above	Between	Below
4 days	*	0	0	*	0	0	*	0	0
	0	*	0	0	*	0	0	*	0
7 days	0	±	0	0	0	0	0	0	0
	±	0	0	0	0	0	0	0	0
16 days	+	±	±	+	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
21 days	++	±	+	0	0	0	0	0	0
	+	+	±	0	0	0	0	0	0
	0	+++	0	0	++	0	0	0	0
	+	+++	+++	+	++	+	++++	0	0
26 days	0	+++	+	0	++	+	0	0	0
	+	+	+	0	+	0	0	0	0
29 days	±	+	0	0	+	0	0	+++	0
	±	++++	++	0	++	+	0	0	++++
38 days	0	+++	*	0	+++	*	0	0	*
	++++	+++	*	+	+++	*	0	0	*

DURATION of LIGATION	BLOOD in LUMEN			BLOOD PIGMENTS in PHAGOCITES			INTEGRITY of INTERNAL ELASTIC LAMELLA		
	Above	Between	Below	Above	Between	Below	Above	Between	Below
4 days	*	YT	+	*	0	0	*	0	0
	+	*	OT	0	*	0	0	*	0
7 days	0	+	±	0	0	0	0	0	0
	+	++	±	0	0	0	0	0	0
16 days	+++	++	+	0	0	0	B	0	0
	++	+	0	0	0	0	0	0	0
21 days	++++	++++	++++	0	0	0	B	B	0
	+	+++	++++	0	0	0	B	B	0
	+++	+	0	0	0	0	0	B	0
	0	±	±	+	0	0	B	0	0
26 days	0	++	+	0	0	0	0	B	0
	+	+	++	0	0	0	B	B	0
29 days	++	+	0	0	+	0	0	0	0
	++	0	+	+	0	+	0	0	0
38 days	++	+	*	0	0	*	B	B	*
	0	0	*	0	0	*	B	B	*

* = Specimen Lost
** = Section through Ligature

N = Nodular Proliferation
C = Concentric Proliferation
NC = Nodular & Concentric Proliferation

0 = no change (e.i. as in control).
± = earliest changes
+, ++, +++, ++++ = arbitrary gradations of degree of change.
//// = complete occlusion.

Y.T = Young thrombus, not organizing.
OT = Organizing Thrombus
B = Broken

V DISCUSSION

It might be well at this point to consider several important questions which the results of this experiment bring out, namely:

I The relationship of our results to those obtained by Baumgarten[84] and by Schaeffer and Radasch.[85]

II The origin of the connective tissue in the proliferated intima.

III The origin of the elastic tissue.

IV The question of the effect of injury.

I It can be seen at once that the results in all three experiments are fundamentally the same, in spite of the more or less minor differences in the techniques employed. In all three experiments, an orderly avascular intimal proliferation was observed between the ligatures, which is distinct from the disorderly vascular fibroblastic proliferation associated with the organization of a thrombus. Baumgarten[84] did not observe any elastic tissue, probably because he did not employ any stain to demonstrate it. Schaeffer and Radasch[85] observed organized thrombus formation between the ligatures, but they do not state at what point between the ligatures, this obser-

vation was made. It will also be remembered that we observed the same condition in two of our 29 day specimens, but concluded from our observation on the one 29 day longitudinal section that the sections showing the organized thrombus were probably taken near the ligature. Baumgarten,[84] in a number of observations on longitudinal sections, observed thrombus formation and subsequent organization in the region of the ligatures, without ever observing this process in the mid-portion between the ligatures.

It is extremely interesting to note that neither Baumgarten,[84] Schaeffer and Radasch,[85] nor ourselves were able to observe any notable disturbance of the media or adventitia, except in the region of the ligatures. This is a particularly significant observation in our series, since we made every effort to isolate the vessel wall from its blood supply.

Finally, the question of the integrity of the internal elastic lamella, following this procedure, should be mentioned. Baumgarten[84] does not mention the presence of any breaks in the internal elastic lamella between the ligatures in his sections. Schaeffer and Radasch[85] state specifically that they did not find any in their rather large series. While it was our observation that they occurred in about half of the specimens taken from between the ligatures, if sufficient sections were taken and careful observations with special stains made.

II There are three schools of thought concerning the origin of the fibroblasts seen early in the intimal proliferation.

Anitschkow,[65] in connection with his work on the feeding of cholesterol to rabbits, observed a fibroblastic proliferation as well as the deposit of lipoid material in the intima; and came to the conclusion that these cells arose from the blood leucocytes, which had emigrated through the endothelium and proceeded to proliferate and undergo a process of differentiation, ultimately becoming fibroblasts. He supports his conclusions by reported observations of leucocytes invading the endothelium and subendothelial tissues from the lumen.

Baumgarten,[84] on the other hand, argues that these cells arise from the endothelial cells by a process of differentiation and proliferation. He supports his contention by stating that he observed many transitional forms in the early proliferation of the endothelium which he consistantly observed in his sections. He further states that he has produced experimentally changes in the endothelium, suggestive of cell metaplasia by painting the wall of a ligated vein with an irritating substance. 24-48 hours following this procedure, he observed a fringe of cells lining the lumen, which possessed all the external qualities of cuboidal epithelium.

Schaeffer and Radasch[85] take a third point of view. They believe that these cells arise from beneath the internal elastic lamella and grow through it - presumably through the fenestrations of Henley, though they do not mention them. They support their contention on the basis of observations on their microscopic sections, in which cells appear to be growing through the internal elastic membrane into the intima.

From our own observations, we are inclined to favor the view of Schaeffer and Radasch.[85] For we had the impression that wē could observe in a number of our sections cells growing from beneath the internal elastic lamella, when it was intact, into the intima. Where there were breaks in the internal elastic lamella, this process was striking. An example of this is shown in Fig. 13.

III The question concerning the origin of the elastic tissue in the intima is also a perplexing one, but there is not the controversy here, probably only because there are not as many logical possibilities concerning its origin. Schaeffer and Radasch[85] state that they believe it is the result of a process of 'protoplasmic activity', but say nothing about the tissue of its origin. Since they found it appearing first from the internal elastic lamella, the assumption is that they are in agreement with the consensus of opinion of other

investigators, namely that this tissue arises in some way or other from the internal elastic lamella.

IV On the basis of the observations of Ssolowjew[81] and others, the question can very justly be raised as to whether the results of these observations are due to the effect of mechanical injury, or to the effect of diminishing the blood supply to the arterial wall. Ramsey,[86] in the course of another experiment in which she exposed the carotid sheath without opening it, doubly ligated the artery and ligated all branches between the ligatures and inserted a canula in the vessel between the ligatures, observed that this procedure had no damaging effect on the wall of the vessel grossly except at the points of the ligature and where the wall was incised. Microscopic sections showed the endothelium to be uniformly intact and closely applied to the internal elastic lamella. The muscular, elastic, and fibrous tissue elements of the media showed no changes. There was no necrosis or hemorrhage in any of the coats and no exudate at any point in the vessel wall proper. Surrounding some of the vessels lying in the connective tissue outside the adventitia were small collections of polymorphonuclear leucocytes, but in no instance were these seen infiltrating the adjacent tissue. From these observations and from the control observations made by Ssolowjew,[81] one may infer that the intimal proliferation observed in this experiment is not due primarily to trauma.

VI CONCLUSIONS

In vessels ligated for a long time there is an intimal thickening which first becomes apparent at the end of one week. The earliest thickening consists of a piling up of endothelium, subsequently a collagenous fibrillar network forms between the endothelium and the internal elastic membrane and, finally, a well formed layer of subendothelial fibrous connective and elastic tissue is layed down. The thickening progresses until the lumen is nearly occluded at the end of five weeks. In the immediate vicinity of the ligatures vascularized connective tissue completely fills the lumen.

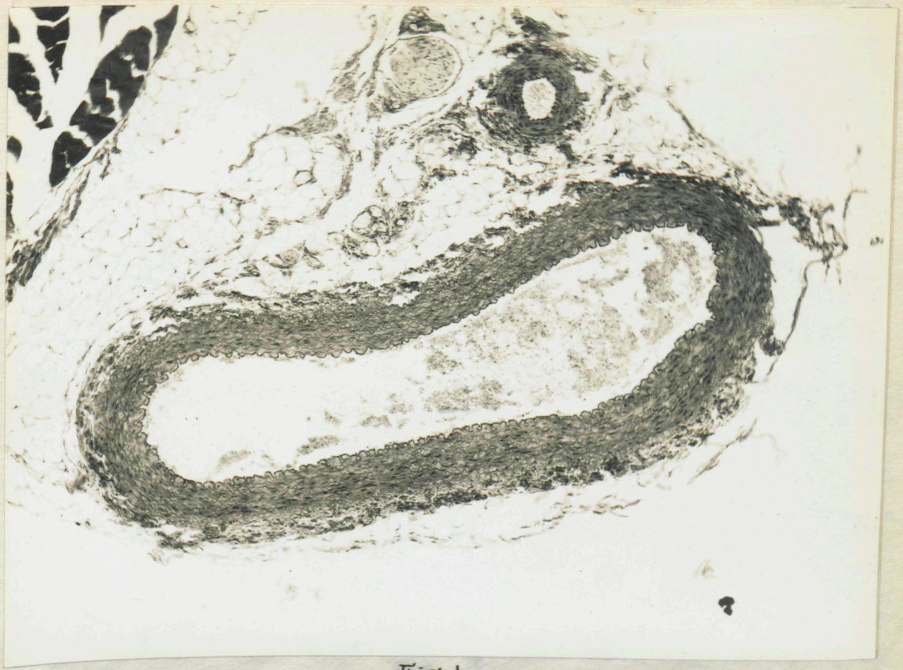


Fig. 1.

#117. Normal Artery. Hemotoxylin-Eosin Stain. x80.

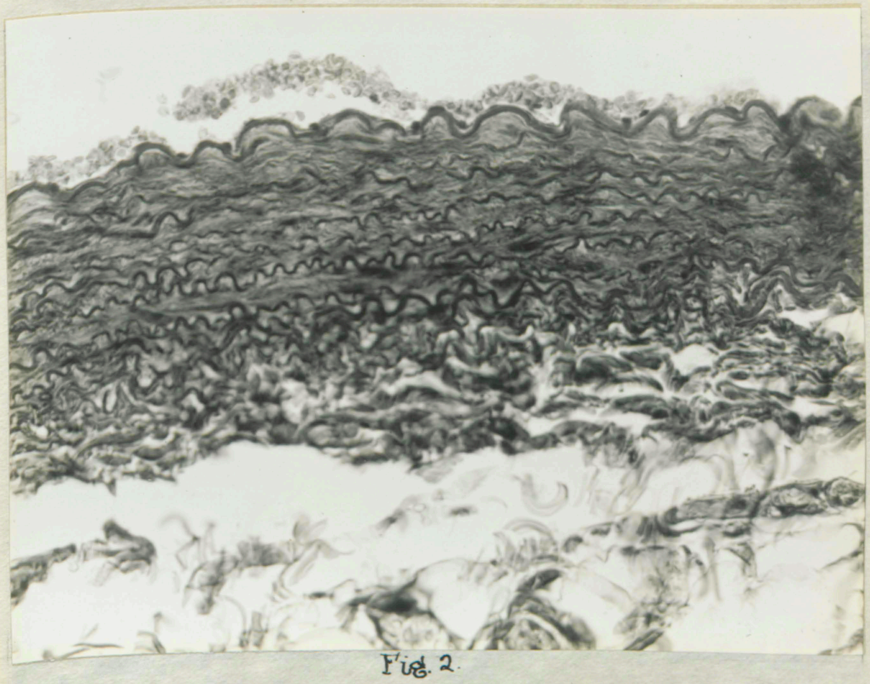


Fig. 2.

#117. Normal Artery. Hemotoxylin-Eosin Stain. x250.

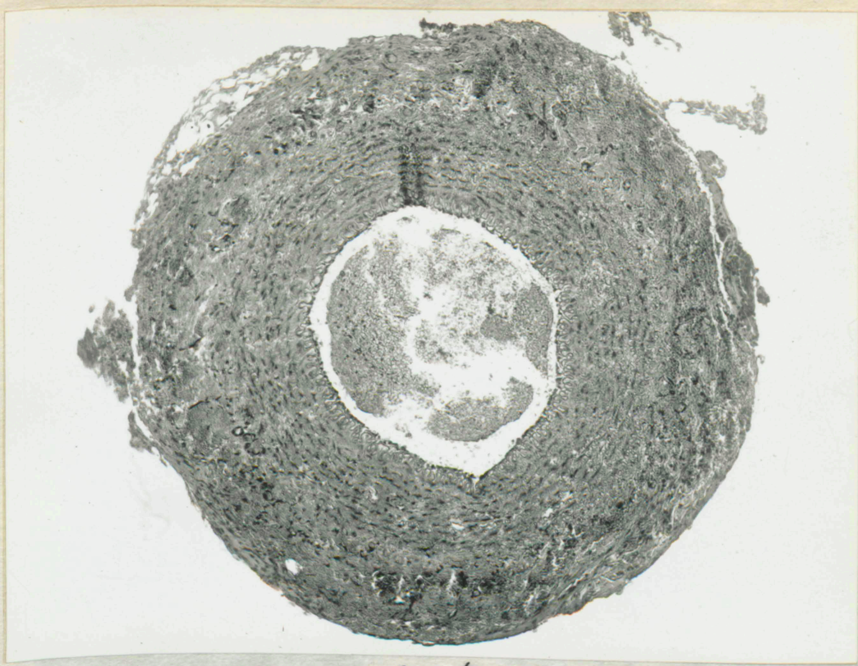


Fig. 3.
#117-3. Double Ligation for 4 days. HemoToxylin-Eosin Stain. X80.

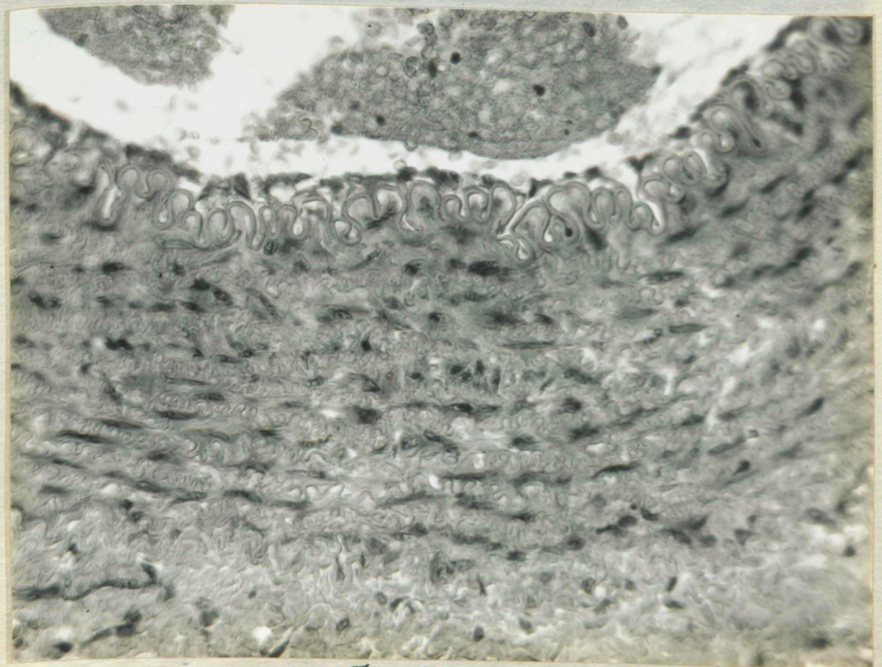


Fig. 4.
#117-3. Double Ligation for 4 days. HemoToxylin-Eosin Stain. X250

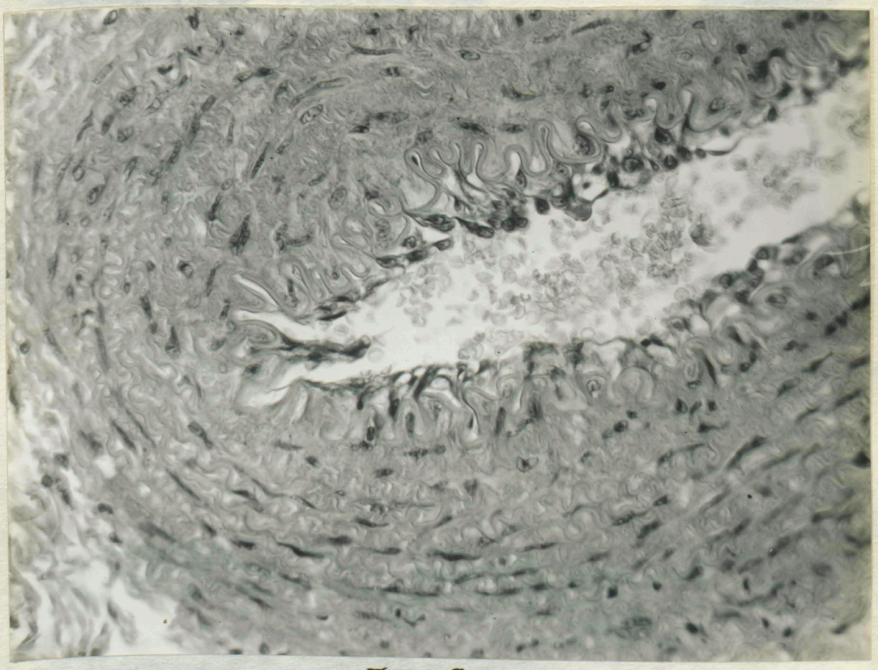


Fig. 5.
115-3. Double Ligation for 7 days. Hemotoxylin-Eosin Stain. x250.

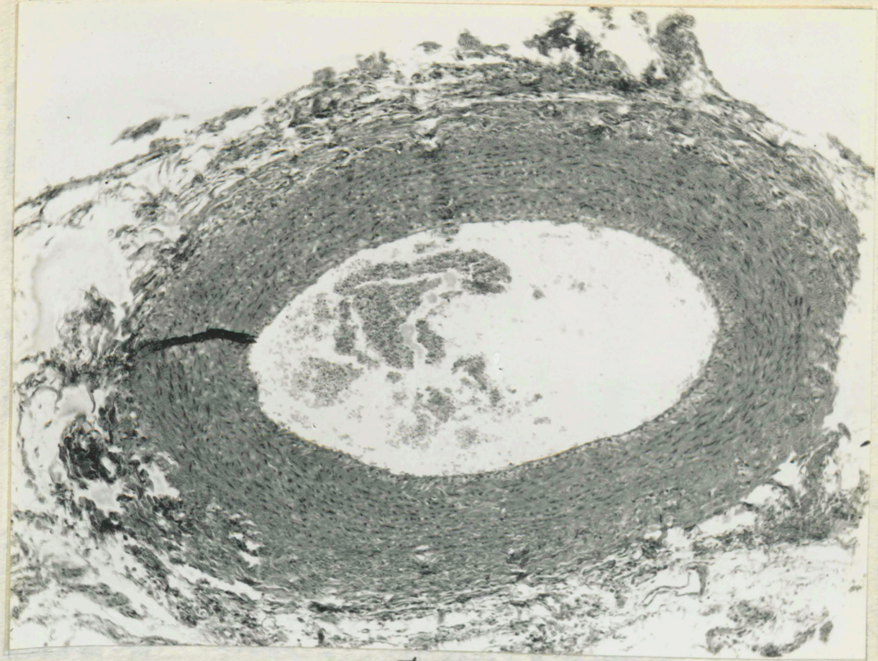


Fig. 6.

#105-3. Double Ligation, for 16 days. Hemotoxylin-Eosin Stain x80.

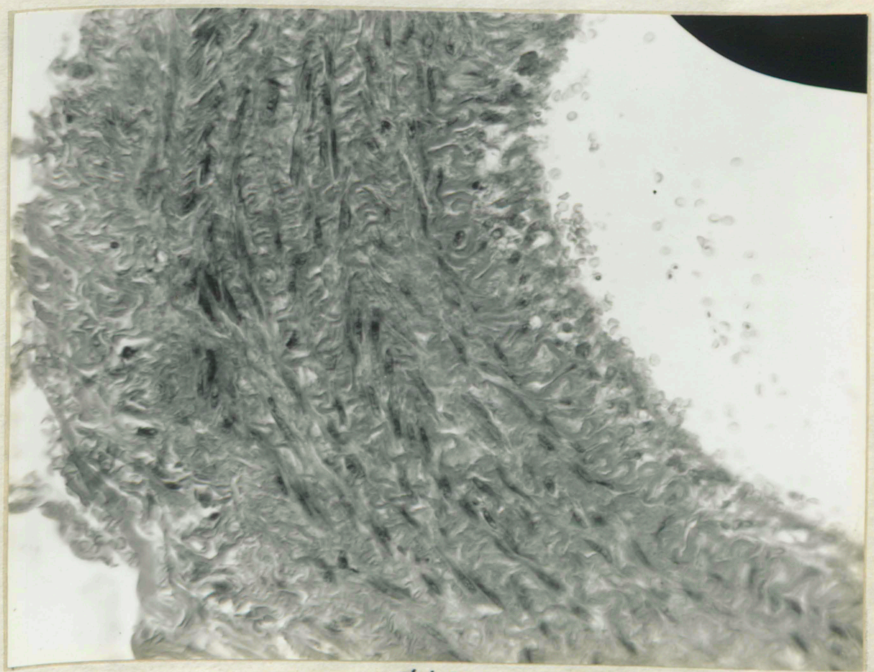


Fig. 7.

#105-3. Double Ligation, for 16 days. Hemotoxylin-Eosin Stain x80.

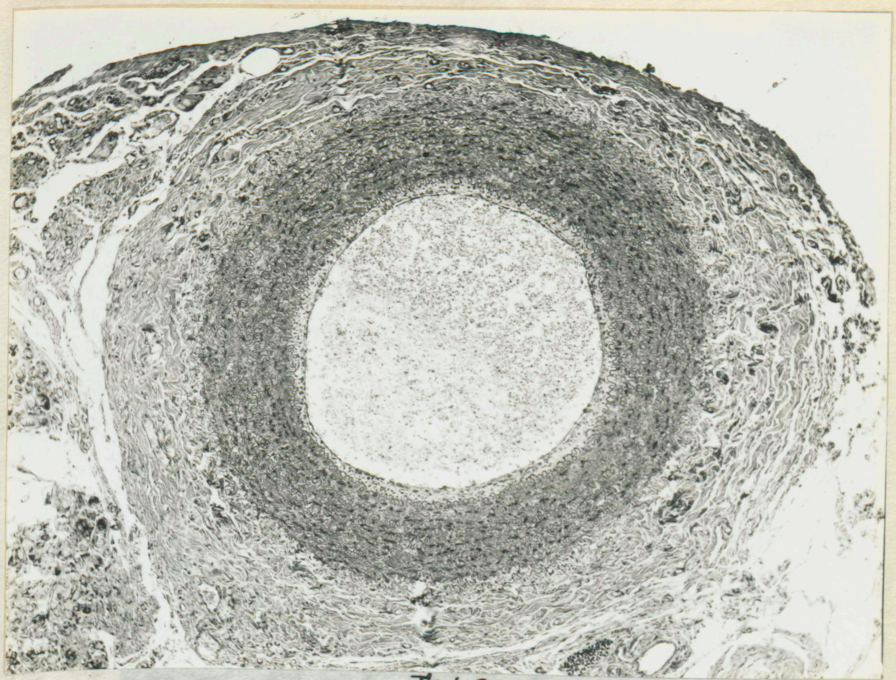


Fig. 8.

#108-3. Double Ligation, for 21 days Hemotoxylin-Eosin Stain. x80.

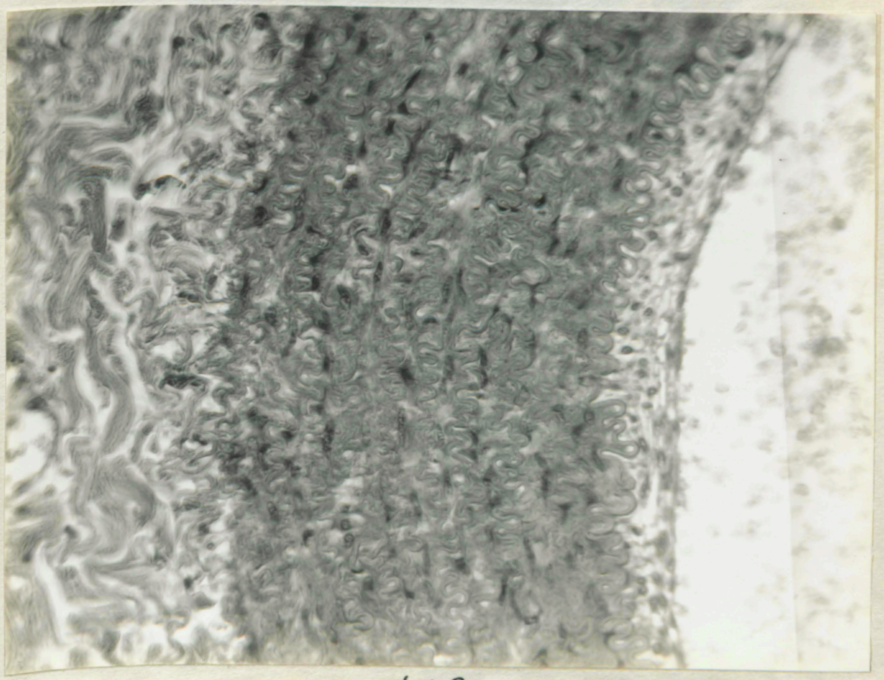


Fig. 9.

#108-3. Double Ligation, for 21 days. Hemotoxylin-Eosin Stain. x250.

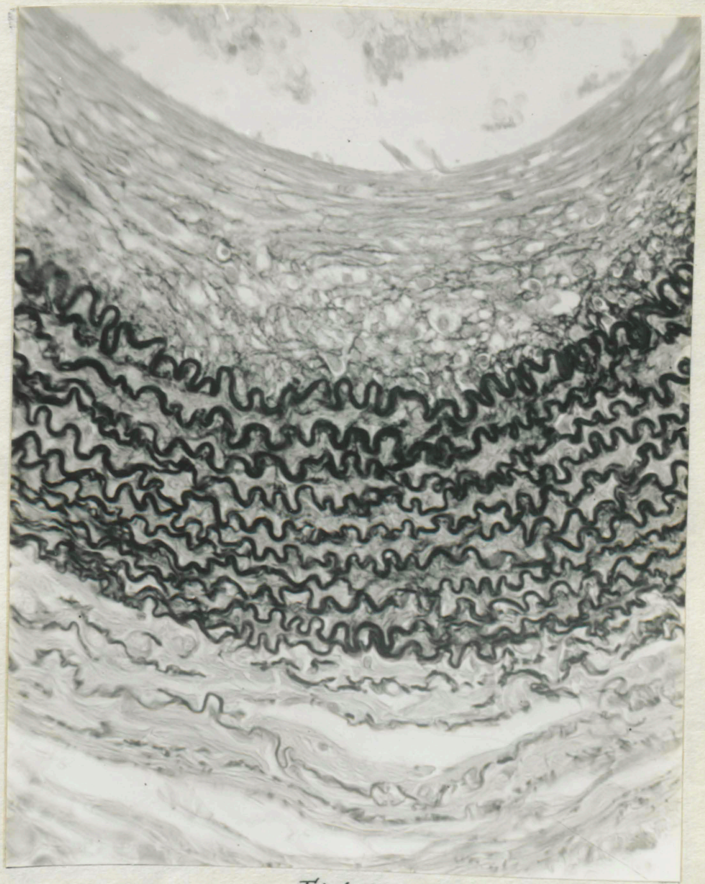


Fig. 10.

#119-3. Double Ligation, for 26 days. Weigert's Elastic Tissue Stain. x 250.

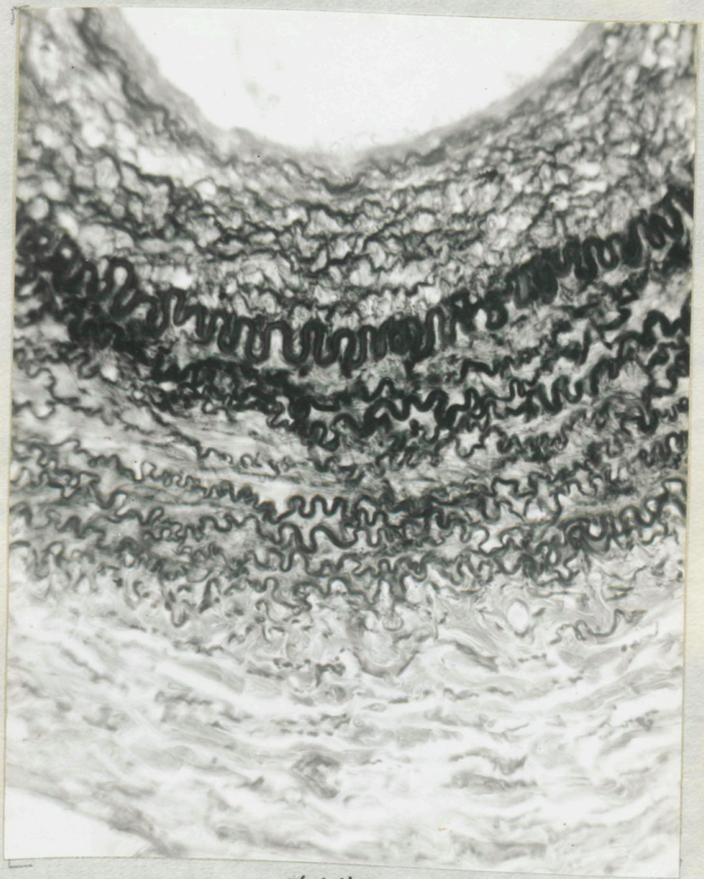


Fig. 11.

#103-2. Double Ligation. for 38 days. Weigert's Elastic Tissue Stain. x250.

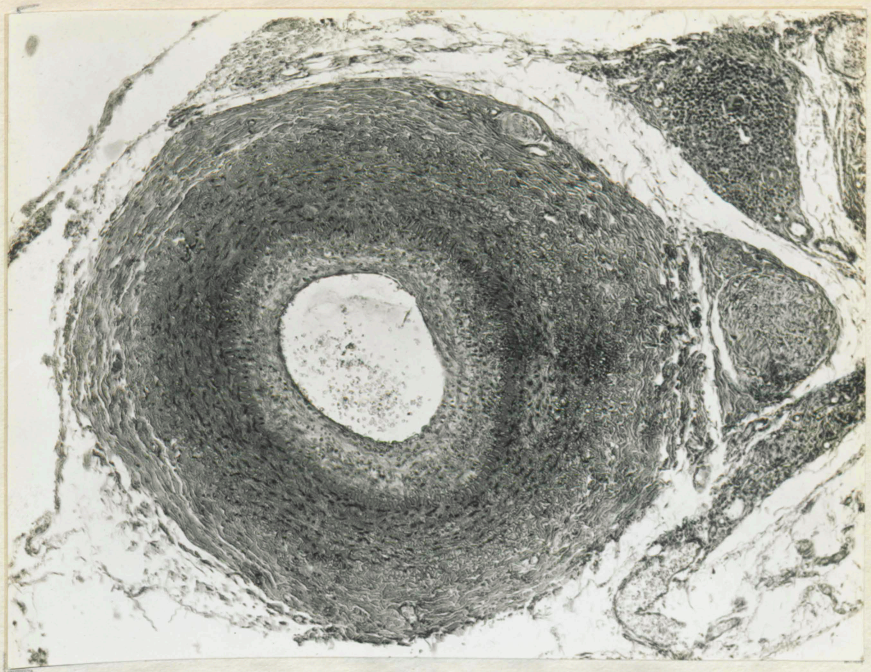


Fig. 12.

#103-2. Double Ligation, for 38 days. Hematoxylin-Eosin Stain x 95.

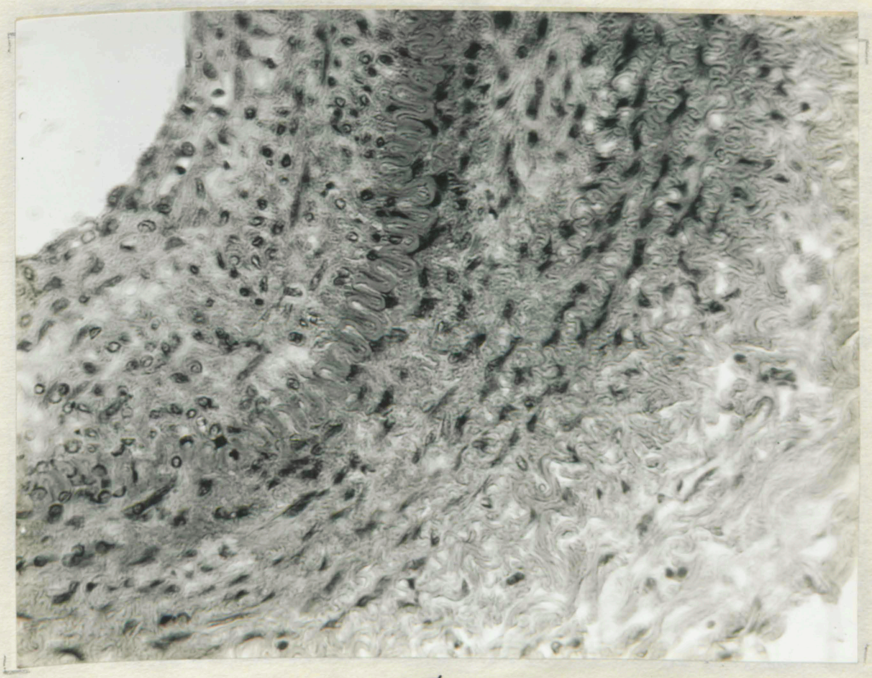


Fig. 13.

#103-2. Double Ligation, for 38 days. Hematoxylin-Eosin Stain. x250.

Fig. 14.

#102-3. Double Ligation, for 29 days. Hematoxylin-Eosin Stain. x 65.

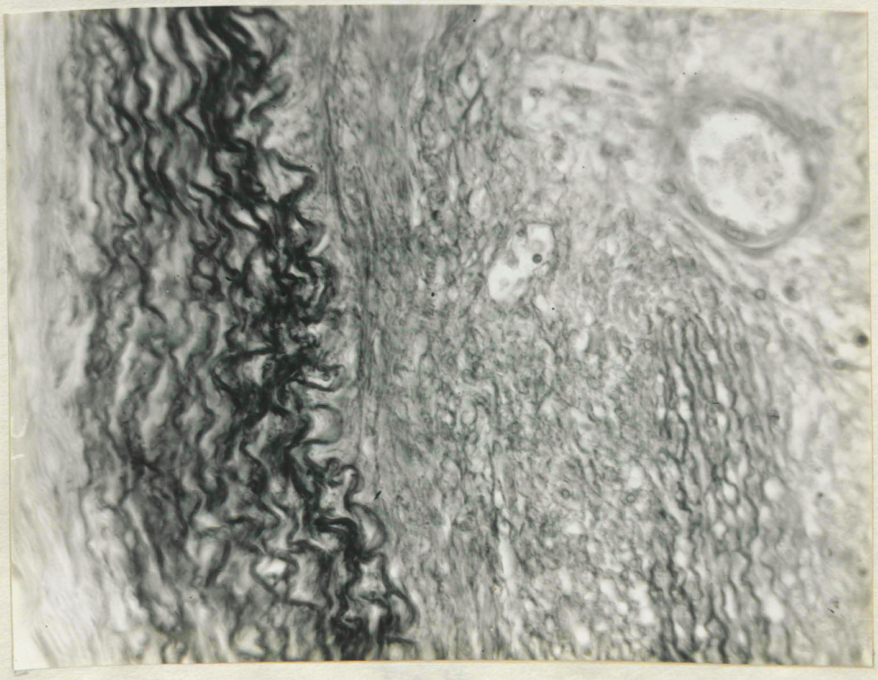


Fig. 15.

#102-3. Double Ligation, for 29 days. Hematoxylin-Eosin Stain. x 250.

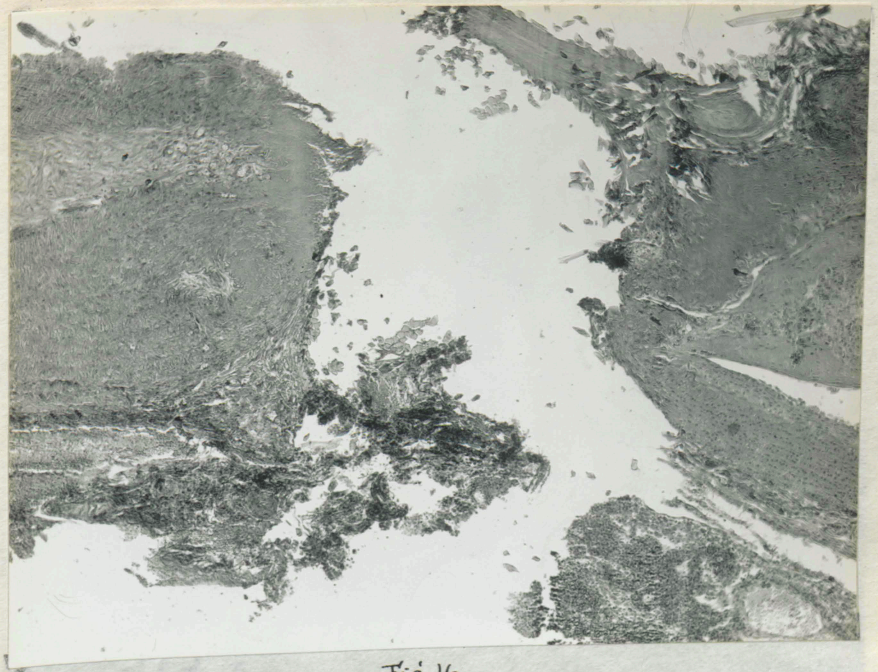


Fig. 16.

Double Ligation for 29 days. Hematoxylin-Eosin Stain. x 65
Longitudinal Section through one of the Ligatures.

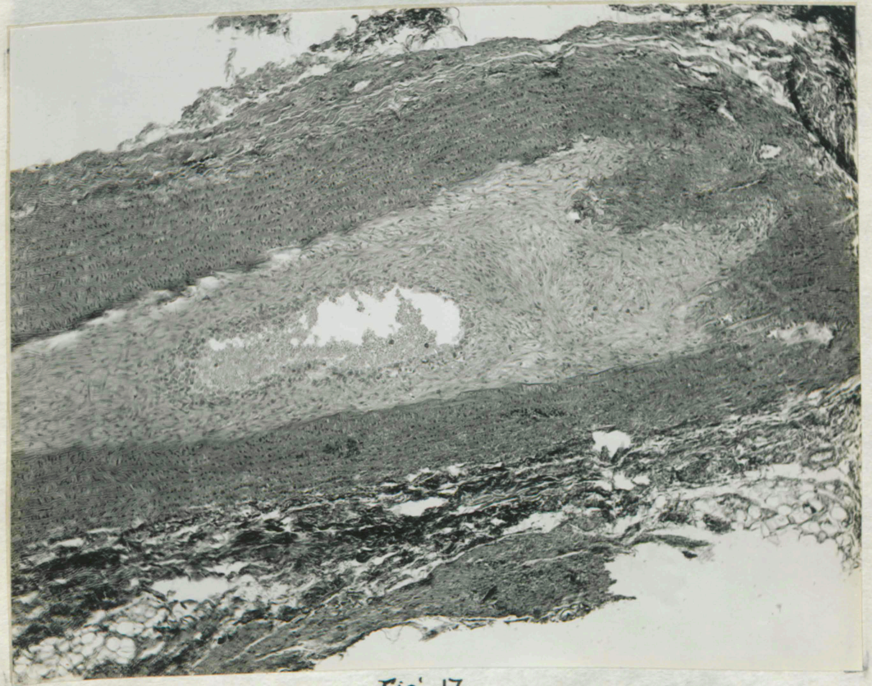


Fig. 17

Double Ligation for 29 days. Hematoxylin-Eosin Stain. x65
Longitudinal Section near the other Ligature.

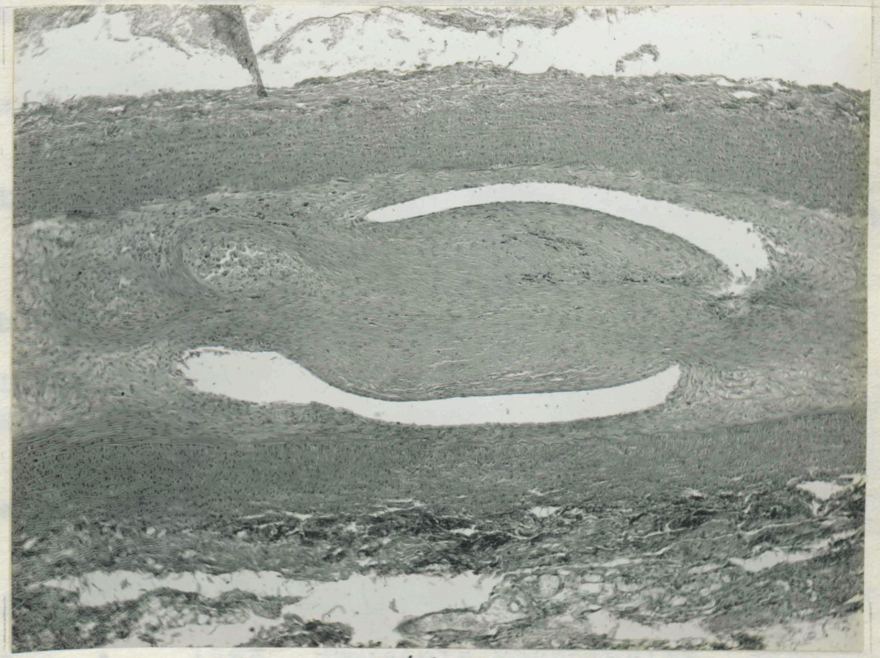


Fig. 18

Double Ligation. for 29 days. Hematoxylin-Eosin Stain. x65.
Longitudinal Section between the ligatures.

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